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Abstract: Objective: Genital use of talcum powder is associated with increased ovarian cancer risk; however, the biologic basis is not completely understood. Epithelial ovarian cancer (EOC) cells manifest a persistent pro-oxidant state. Our objective was to determine the effects of talc on expression of key inflammatory and redox markers in ovarian cancer and normal cell lines.
Methods: Normal ovarian and EOC cells were treated with various doses of talc for 48 hours. Levels of CA-125 and selected key redox enzymes, including myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX1) and glutathione reductase (GSR) were determined with real-time RT-PCR and ELISA assays. TaqMan® Genotype analysis utilizing the QuantStudio 12K Flex was used to assess single nucleotide polymorphisms (SNPs). Data was analyzed with one-way ANOVA followed by Tukey's post hoc tests with Bonferroni correction.
Results: In all talc treated cells, there was a significant dose-dependent increase in iNOS, nitrate/nitrite, and MPO (pro-oxidants) with a concomitant decrease in CAT, SOD3, GSR, and GPX (antioxidants) ($p<0.05$). There also was a significant increase in CA-125 in all talc treated cells, except macrophages ($p<0.05$). Furthermore, we identified an induction of specific mutations in these key enzymes that correlated with alterations of their activities in talc treated cells compared to their controls.
Conclusion: Talcum powder enhanced the redox state in normal ovarian and EOC cells, providing a molecular basis to previous reports linking its genital use to increased ovarian cancer risk.

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Beth Y. Karlan, M.D.
Editor-in-Chief
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August 22, 2018

Dear Dr. Karlan,

Enclosed is the manuscript entitled, "Molecular basis supporting the association of talcum powder use with increased risk of ovarian cancer." We appreciate your consideration for its publication as a research paper in *Gynecologic Oncology*. The use of talcum powder and ovarian cancer risk has been associated by many investigators; however, the molecular mechanism by which this occurs has not been substantiated. Using our previously published research associating modulation of redox balance within the ovarian epithelium and the development of ovarian cancer, we studied the effect of talc on the redox state of normal ovarian epithelium, fallopian tube epithelium, and ovarian cancer cell lines. We were able to correlate altered expression of key pro-oxidant and antioxidants to specific single nucleotide polymorphisms within these cell lines, thus indicating a molecular mechanism of talcum powder in the development of the oncogenic phenotype.

This is a novel finding and the first of its kind to be reported. We believe that such groundbreaking findings will appeal to the readers of Gynecologic Oncology and serve as a catalyst for future studies. Each of the authors has contributed a substantial amount of work to the development, experiments, and/or reporting of this research. Each confirms that this manuscript has not been previously published and is not under consideration by any other journal. All authors have reviewed and approve of the content of this manuscript and have no conflicts of interest. We greatly appreciate your consideration for review and publication.

Sincerely,

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Molecular basis supporting the association of talcum powder use with increased risk of ovarian cancer.

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Abstract

Objective: Genital use of talcum powder is associated with increased ovarian cancer risk; however, the biologic basis is not completely understood. Epithelial ovarian cancer (EOC) cells manifest a persistent pro-oxidant state. Our objective was to determine the effects of talc on expression of key inflammatory and redox markers in ovarian cancer and normal cell lines.

Methods: Normal ovarian and EOC cells were treated with various doses of talc for 48 hours. Levels of CA-125 and selected key redox enzymes, including myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) were determined with real-time RT-PCR and ELISA assays. TaqMan® Genotype analysis utilizing the QuantStudio 12K Flex was used to assess single nucleotide polymorphisms (SNPs). Data was analyzed with one-way ANOVA followed by Tukey's post hoc tests with Bonferroni correction.

Results: In all talc treated cells, there was a significant dose-dependent increase in iNOS, nitrate/nitrite, and MPO (pro-oxidants) with a concomitant decrease in CAT, SOD3, GSR, and GPX (antioxidants) ($p<0.05$). There also was a significant increase in CA-125 in all talc treated cells, except macrophages ($p<0.05$). Furthermore, we identified an induction of specific mutations in these key enzymes that correlated with alterations of their activities in talc treated cells compared to their controls.

Conclusion: Talcum powder enhanced the redox state in normal ovarian and EOC cells, providing a molecular basis to previous reports linking its genital use to increased ovarian cancer risk.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and ranks fifth in cancer deaths among women diagnosed with cancer [1]. Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome [1, 2]. It comprises at least five distinct histological subtypes, the most common and well-studied being high-grade serous ovarian cancer. Although surgical techniques and treatments have advanced over the years, the prognosis of EOC remains poor, with a 5-year survival rate of 50% in advanced stage [2]. This is largely due to the lack of early warning symptoms and screening methods, and the development of chemoresistance [1, 2]. Moreover, ovarian cancer is known to be associated with germline mutations in the BRCA1 or BRCA2 genes, but with a rate of only 20-40%, this suggests the presence of other unknown mutations in other predisposition genes [3]. Additional genetic variations including single nucleotide polymorphisms (SNPs) have been hypothesized to act as low to moderate penetrant alleles that contribute to ovarian cancer risk [3, 4]. Non-synonymous SNPs substitute encoded amino acids in proteins and are more likely to alter the structure, function, and interaction of the protein [4].

The pathophysiology of EOC is not fully understood but has been strongly associated with inflammation and the resultant oxidative stress [5]. We have previously characterized EOC cells to manifest a persistent pro-oxidant state as evident by the upregulation of key oxidants and downregulation of key antioxidants. This redox state is further enhanced in chemoresistant EOC cells [6]. The expression of key pro-oxidant/inflammatory enzymes such as inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, and myeloperoxidase (MPO), as well as an increase in nitric oxide (NO) levels were increased in EOC tissues and cells [7]. Additionally, we have shown that EOC cells manifest lower apoptosis, which was markedly induced by inhibiting iNOS, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [7].

The cellular redox balance is maintained by key antioxidants including catalase (CAT), superoxide dismutase (SOD) or by glutathione peroxidase (GPX) coupled with glutathione reductase

(GSR) [5]. Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate [8]. We have previously reported that a genotype switch in key antioxidants is a potential mechanism leading to the acquisition of chemoresistance in EOC cells [9]. We have studied the effects of genetic polymorphisms in key redox genes on the acquisition of the oncogenic phenotype in EOC cells. Functional polymorphisms in genes that control the levels of cellular ROS and oxidative damage, including SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways [4, 9] were tested. Several function-altering SNPs have been identified in key antioxidants, including CAT, GPX, GSR, and SOD3 [4].

Several studies have suggested the possible association between genital use of talcum powder and risk of EOC [10-12] . Association between the use of cosmetic talc in genital hygiene and ovarian cancer was first described in 1982 by Cramer, et al, and many subsequent studies found talc use to increase risk for ovarian cancer [13]. Talc and asbestos are both silicate minerals; the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature [12, 14]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response [12]. The objective of this study was to determine the effects of talcum powder on the expression of key redox enzymes and CA-125 levels in normal and EOC cells.

Material and Methods

Cell Lines: Ovarian cancer cells: SKOV-3 (ATCC), A2780 (Sigma Aldrich, St. Louis, MO), and TOV112D (a kind gift from Gen Sheng Wu at Wayne State University, Detroit, MI). Normal cell lines: human macrophage cells (EL-1, ATCC, Manassas, VA), human primary normal ovarian epithelial cells (Cell Biologics, Chicago, IL), human ovarian epithelial cells (HOSEpiC, ScienCell Research Laboratories, Inc., Carlsbad, CA), immortalized human fallopian tube secretory epithelial cells (FT33-shp53-R24C, Applied Biological Materials, Richmond, BC, Canada). All cells were grown in media and

conditions following manufacturer's protocol. EL-1 cells were grown in IMDM media (ATCC) supplemented with 0.1 mM hypoxanthine and 0.1 mM thymidine solution (H-T, ATCC) and 0.05mM β-mercaptoethanol. SKOV-3 EOC cells were grown in HyClone McCoy's 5A medium (Fisher Scientific, Waltham, MA), A2780 EOC cells were grown in HyClone RPMI-1640 (Fisher Scientific), and both TOV112D EOC cells were grown in MCDB105 (Cell Applications, San Diego, CA) and Medium 199 (Fisher Scientific) (1:1). All media was supplemented with fetal bovine serum (FBS, Innovative Research, Novi, MI) and penicillin/streptomycin (Fisher Scientific), per their manufacturer specifications. Human primary normal ovarian epithelial cells were grown in Complete Human Epithelial Cell Medium (Cell Biologics).

Treatment of cells: Talcum powder (Fisher Scientific, Catalog #T4-500, Lot#166820) or baby powder (Johnson & Johnson, #30027477, Lot#13717RA) was dissolved in DMSO (Sigma Aldrich) at a concentration of 500 mg in 10 ml and was filtered with a 0.2 µm syringe filter (Corning). Sterile DMSO was used as a control for all treatments. Cells were seeded in 100 mm cell culture dishes (3×10^6) and were treated 24 hours later with 100 µg/ml of talc for 48 hours. Cell pellets were collected for RNA, DNA, and protein extraction. Cell culture media was collected for CA-125 analysis by ELISA.

Real-time RT-PCR: Total RNA was extracted from all cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the protocol provided by the manufacturer. Measurement of the amount of RNA in each sample was performed using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A 20 µL cDNA reaction volume containing 0.5 µg RNA was prepared using the SuperScript VILO Master Mix Kit (Life Technologies, Carlsbad, CA), as described by the manufacturer's protocol. Optimal oligonucleotide primer pairs were selected for each target using Beacon Designer (Premier Biosoft, Inc., S1). Quantitative RT-PCR was performed using the EXPRESS SYBR GreenER qPCR Supermix Kit (Life Technologies) and the Cepheid 1.2f Detection System as previously described [6]. Standards with known concentrations and lengths were designed specifically for *β-actin* (79 bp), *CAT* (105 bp), *NOS2* (89 bp), *GSR* (103 bp), *GPX1* (100 bp), *MPO* (79 bp), and *SOD3* (84 bp), allowing for

construction of a standard curve using a 10-fold dilution series [6]. A specific standard for each gene allows for absolute quantification of the gene in number of copies, which can then be expressed per microgram of RNA. All samples were normalized to the housekeeping gene, *β-actin*. A final melting curve analysis was performed to demonstrate specificity of the PCR product.

Protein Detection: Cell pellets were lysed utilizing cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin) containing a cocktail of protease inhibitors. Samples were centrifuged at 13000 rpm for 10 minutes at 4°C. Total protein concentration of cell lysates from control and talc treated cells was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) per the manufacturer's protocol.

Detection of protein/activity by ELISA: ELISA kits for each target were purchased and used according to the manufacturer's protocol. The following ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI): CAT, SOD3, GSR, GPX, and MPO. Nitrite (NO_2^-)/nitrate (NO_3^-) were determined spectrophotometrically by measuring their absorbance at 210 nm after separation by HPLC with standard NO_2^-/NO_3^- as previously reported [6]. The analysis was performed by a HPLC system (Shimadzu Scientific Instruments, Inc.) including a LC-10ADV pump, frc-10A injector and DGU-14A degasser. Nitrite/nitrate was detected using an RF-10 XL fluorescence detector with 210 nm excitation and 440 nm emission. CA-125 protein levels were measured in cell media by ELISA from Ray Biotech (Norcross, GA) according to the manufacturer's protocol.

TaqMan® SNP Genotyping Assay: DNA was isolated utilizing the EZ1 DNA Tissue Kit (Qiagen) for EOC cells according the manufacturer's protocols. The TaqMan® SNP Genotyping Assay Set (Applied Biosystems, Carlsbad, CA) (NCBI dbSNP genome build 37, MAF source 1000 genomes) were used to genotype the SNPs (S1). The Applied Genomics Technology Center (AGTC, Wayne State University, Detroit, MI) performed these assays. Analysis was done utilizing the QuantStudioTM 12 K Flex Real-Time PCR System (Applied Biosystems).

Statistical Analysis: Normality was examined using the Kolmogorov-Smirnov test and by visual inspection of quantile-quantile plots. Because most of the data were not normally distributed, differences in distributions were examined using the Kruskal-Wallis test. Generalized linear models were fit to examine pairwise differences in estimated least squares mean expression values by exposure to 0, 5, 20 or 100 µg/ml of talc. We used the Tukey-Kramer adjustment for multiple comparisons, and the regression models were fit using log2 transformed analyte expression values after adding a numeric constant '1' to meet model assumptions while avoiding negative transformed values. P-values below 0.05 are statistically significant.

Results

Talcum powder treatment decreased the expression of antioxidant enzymes SOD and CAT in normal and EOC cells. Real-time RT-PCR and ELISA assays were utilized to determine the CAT and SOD mRNA and protein levels in cells before and after 48 hours talc treatment, respectively (Figure 1). CAT mRNA and protein levels were significantly decreased in a dose-dependent manner in talc treated cells compared to controls (Figure 1. A, C, $p<0.05$). Similarly, SOD mRNA and protein levels were significantly decreased in a dose- dependent manner in talc treated cells compared to controls (Figure 1. B, D, $p<0.05$).

Talcum powder treatment increased the expression of prooxidants iNOS, NO_2^-/NO_3^- and MPO in normal and EOC cells. Real-time RT-PCR and NO_2^-/NO_3^- assays were utilized to determine the iNOS mRNA and NO levels in cells before and after 48 hours talc treatment, respectively (Figure 2). iNOS mRNA and NO levels were significantly increased in a dose-dependent manner in talc treated cells as compared to their controls (Figure 2. A, C, $p<0.05$). As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus talc treatment did not have any effect. However, MPO mRNA and protein levels were significantly increased in a dose-dependent manner in talc treated ovarian cancer cells and macrophages compared to controls (Figure 2. B, D, $p<0.05$).

Talcum powder treatment decreased the expression of antioxidant enzymes, GPX and GSR in

normal and EOC cells. Real-time RT-PCR and ELISA assays were utilized to determine the GPX and GSR mRNA and protein levels in cells before and after 48 hours talc treatment, respectively (Figure 3). GPX mRNA and protein levels were significantly decreased in a dose-dependent manner in talc treated cells compared to controls (Figure 3. A, C, $p<0.05$). Similarly, GSR mRNA and protein levels were significantly decreased in a dose-dependent manner in talc treated cells compared to controls (Figure 3. B, D, $p<0.05$).

Talcum powder treatment increased CA-125 levels in normal and EOC cells. CA-125 ELISA assay was performed in protein isolated from cell media before and after talc treatment. CA-125 levels were significantly increased in a dose-dependent manner in all cells (Figure 4, $p<0.05$). There was no detectable CA-125 protein in macrophages.

Talc exposure induced known genotype switches in key oxidant and antioxidant enzymes. Talc treatment was associated with a genotype switch in *NOS2* from the common C/C genotype in untreated cells to T/T, the SNP genotype, in talc treated cells, except in A2780 and TOV112D (Table 1). Additionally, the observed decrease in CAT expression and activity was associated with a genotype switch from common C/C genotype in CAT in untreated cells to C/T, the SNP genotype, in TOV112D and all normal talc treated cells. However, there was no detectable genotype switch in CAT in A2780, SKOV3, and TOV112D (Table 1). Remarkably, there was no observed genotype switch in the selected SNP for SOD3 and GSR in all talc treated cells. All cells except for HOSEpiC cells manifest the SNP genotype of *GPX1* (C/T). Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 1).

Discussion

The claim that regular use of talcum powder for hygiene purpose is associated with an increased risk of ovarian cancer is based on several reports confirming the presence of talc particles in the ovaries and other parts of the female reproductive tract as well as in lymphatic vessels and tissues of the pelvis

[10, 12]. A study by Cramer, et al has reported the presence of talc in pelvic lymph nodes of a woman with ovarian cancer who used talc daily for 30 years [10]. The ability of talc particles to migrate through the genital tract to the distal fallopian tube and ovaries is well accepted [11, 15]. To date, the exact mechanism is not fully understood, though several studies have pointed toward the peristaltic pump feature of the uterus and fallopian tubes, which is known to enhance transport of sperm into the oviduct ipsilateral to the ovary bearing the dominant follicle [16-18].

There are reports supporting the epidemiologic association of talc use and risk of ovarian cancer [19, 20]. Recent studies have shown that risks for EOC from genital talc use vary by histologic subtype, menopausal status at diagnosis, hormone therapy use, weight, and smoking. These observations suggest that estrogen and/or prolactin may play a role via macrophage activity and inflammatory response to talc. There has been debate as to the significance of the epidemiologic studies based on the fact that the reported epidemiologic risk of talc use and risk of ovarian cancer, although consistent, is relatively modest (30-40%), and there is inconsistent increase in risk with duration of use. This observation is due, in part, to the challenges in quantifying exposure as well as the failure of epidemiological studies to obtain necessary information about the frequency and duration of usage [19-21].

In this study, we have shown beyond doubt that talcum powder alters key redox and inflammatory markers, a hallmark of ovarian cancer. More importantly, this also occurs in normal cells, including surface ovarian epithelium, fallopian tube, and macrophages. Oxidative stress has been implicated in the pathogenesis of ovarian cancer, specifically, by increased expression of several key pro-oxidant enzymes such as iNOS, MPO, and NAD(P)H oxidase in EOC tissues and cells as compared to normal cells indicating an enhanced redox state, as we have recently demonstrated [7]. This redox state is further enhanced in chemoresistant EOC cells as evident by a further increase in iNOS and $NO2^-/NO3^-$ and a decrease in GSR levels, suggesting a shift towards a pro-oxidant state [7]. Antioxidant enzymes, key regulators of cellular redox balance, are differentially expressed in various cancers, including ovarian [7, 22]. Specifically, GPX expression is reduced in prostate, bladder, and estrogen receptor negative

breast cancer cell lines as well as in cancerous tissues from the kidney. However, GPX activity is increased in cancerous tissues from breast [22]. Glutathione reductase levels, on the other hand, are elevated in lung cancer, although differentially expressed in breast and kidney cancerous tissues [5, 23]. Similarly, CAT was decreased in breast, bladder, and lung cancer while increased in brain cancer [24-26] Superoxide dismutase is expressed in lung, colorectal, gastric ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells [26-29] Collectively, this differential expression of antioxidants demonstrates the unique and complex redox microenvironment in cancer. Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. This enzyme is essential for the GSH redox cycle which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress (Figure 5). Treatment with talc significantly reduced GSR in normal and cancer cells, altering the redox balance (Figure 3. A, C). Likewise, GPX is an enzyme that detoxifies reactive electrophilic intermediates and thus plays an important role in protecting cells from cytotoxic and carcinogenic agents. Overexpression of GPX is triggered by exogenous chemical agents and reactive oxygen species, and is thus thought to represent an adaptive response to stress [23]. Indeed, treatment of normal and cancer cells with talc significantly reduced GPX, which compromised the overall cell response to stress (Figure 3. B, D).

We have previously reported a cross-talk between iNOS and MPO in ovarian cancer which contributed to the lower apoptosis observed in ovarian cancer cells [7, 30]. Myeloperoxidase, an abundant hemoprotein, previously known to be present solely in neutrophils and monocytes, is a key oxidant enzyme that utilizes NO produced by iNOS as a one-electron substrate generating NO^+, a labile nitrosylating species [7, 31, 32]. Indeed, we were the first to report that MPO was expressed by EOC cells and tissues [30]. Silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S-nitrosylation of caspase-3 by MPO [30]. Additionally, we have compelling evidence that MPO serves as a source of free iron under oxidative

stress, where both NO^+ and superoxide are elevated [7]. Iron reacts with hydrogen peroxide (H_2O_2) and catalyzes the generation of highly reactive hydroxy radical ($HO^\bullet$), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber–Weiss reaction [7, 32]. We have previously highlighted the potential benefits of the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer [33]. Collectively, we now have substantial evidence demonstrating that altered oxidative stress may play a role in maintaining the oncogenic phenotype of EOC cells. Treatment of normal or ovarian cancer cells with talc resulted in a significant increase in MPO and iNOS, supporting the role of talc in the enhancement of a pro-oxidant state that is a major cause in the development and maintenance of the oncogenic phenotype (Figure 2).

Furthermore, CA-125, which exists as a membrane-bound and secreted protein in epithelial ovarian cancer cells, has been established as a biomarker for disease progression and response to treatment [2]. CA-125 expression was significantly increased from nearly undetectable levels in controls to values approaching clinical significance (35 U/ml in postmenopausal women [34]) in talc treated cell lines (Figure 4, $p<0.05$) without the physiologic effects on the tumor microenvironment one would expect to be present in the human body, highlighting the implications of the pro-oxidant states caused by talc alone.

To elucidate the mechanism by which talcum powder alters the redox balance to favor a pro-oxidant state not only in ovarian cancer cells, but more importantly in normal cells, we have examined selected known gene mutations in key oxidant and antioxidant enzymes. These mutations correspond to specific SNPs that are known to be associated with altered enzymatic activity and increased cancer risk [6, 9]. Our results show that the *CAT* SNP (rs769217) which results in decreased enzymatic activity was induced in all normal cell lines tested and in TOV112D EOC lines. However, the *CAT* mutation was not detected in A2780 or SKOV-3 cell lines (Table 1). Nevertheless, our results confirm a decrease in CAT expression and enzymatic activity in all talc treated cells (Figure 1), indicating the existence of other *CAT* SNPs. However, the *SOD3* (rs2536512) and *GSR* (rs8190955) SNP genotypes were not detected in any

cell line, yet SOD3 and GSR activity and expression were decreased in all talc treated cells, again suggesting the presence of other SNPs. Our results have also shown that all cells, except for HOSEpiC cells, manifest the SNP genotype of *GPX1* (C/T) before talc treatment. Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 1). Consistent with this finding, we have previously reported that acquisition of chemoresistance by ovarian cancer cells is associated with a switch from the *GPX1* SNP genotype to the normal *GPX1* genotype [6]. It is not understood why a *GPX1* SNP genotype predominates in untreated normal and ovarian cancer cells. Additionally, our results showed that talc treatment was associated with a genotype switch from common C/C genotype in *NOS2* in untreated cells to T/T, the SNP genotype, in talc treated cells, except in A2780 and TOV112D (Table 1). Nevertheless, our results confirm an increase in iNOS expression and enzymatic activity in all talc treated cells (Figure 2), again suggesting the existence of other *NOS2* SNPs. Collectively, these findings support the notion that talc treatment induced gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for the initiation and development of ovarian cancer. Future studies examining such SNPs are important to fully elucidate a genotype switch mechanism induced by talcum powder exposure.

In summary, this is the first study to clearly demonstrate that talcum powder induces inflammation and alters the redox balance favoring a pro-oxidant state in normal and EOC cells. We have shown a dose-dependent significant increase in key pro-oxidants, iNOS, $NO2^-/NO3^-$, and MPO and a concomitant decrease in key antioxidant enzymes, CAT, SOD, GPX, and GSR, in all talc treated cells (both normal and ovarian cancer) compared to their controls. Additionally, there was a significant increase in CA-125 levels in all the talc treated cells compared to their controls, except in macrophages. The mechanism by which talc alters the cellular redox and inflammatory balance involves the induction of specific mutations in key oxidant and antioxidant enzymes that correlate with alterations in their activities. The fact that these mutations happen to correspond to known SNPs of these enzymes indicate a genetic predisposition to developing ovarian cancer with genital talcum powder use.

Conflict of Interest

The authors have no conflicts of interest to declare.

Author Contributions

Dr. Nicole Fletcher significantly contributed to the design of this study and acquisition of data, participated in the drafting and revision of the article, and gave final approval of the submitted manuscript.

Ira Memaj significantly contributed to the acquisition of data, participated in the drafting and revision of the article, and gave final approval of the submitted manuscript.

Rong Fan significantly contributed to the acquisition of data, participated in the drafting and revision of the article, and gave final approval of the submitted manuscript.

Dr. Amy Harper significantly contributed to the analysis and interpretation of data, provided clinical correlation of data, participated in the drafting and revision of the article, and gave final approval of the submitted manuscript.

Dr. Robert Morris significantly contributed to the analysis and interpretation of data, participated in the drafting and revision of the article, provided clinical expertise, and gave final approval of the submitted manuscript.

Dr. Ghassan Saed served as the principal investigator of this study and significantly contributed to its conception and design, analysis and interpretation of data, participated in the drafting and revision of the article, and gave final approval of the submitted manuscript.

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Table Legends:

Table 1. SNP characteristics (A) and SNP genotyping of key redox enzymes in untreated and talc treated (100 μg/mL) human primary ovarian epithelial cells (Normal ovarian), Human Ovarian Surface Epithelial Cells (HOSEpiC) , fallopian tube (FT33), and ovarian cancer (A2780, SKOV-3, TOV112D) cell lines (B).

Figure Legends:

Figure 1. Decreased expression and activity of key antioxidant enzymes, CAT and SOD3. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of CAT (A&C) and SOD3 (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 48 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

Figure 2. Increased expression and activity of key pro-oxidants, iNOS, NO_2^-/NO_3^-, and MPO. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of iNOS (A&C) and MPO (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 48 hours. As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus talc treatment did not have any effect. Experiments were performed in triplicate. Expression is depicted as the mean with

error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in iNOS and MPO positive cells and in all doses as compared to controls.

Figure 3. Decreased expression and activity of key antioxidant enzymes, GSR and GPX. The mRNA (real-time RT-PCR) and protein/activity levels (ELISA) of GSR (A&C) and GPX (B&D) were determined in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 48 hours. Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells and in all doses as compared to controls.

Figure 4. Increased CA125 levels in response to talc treatment. The level of ovarian cancer biomarker CA-125 was determined by ELISA before and after 48 hours of talc treatment (100 µg/ml) in macrophages (EL-1), human primary ovarian epithelial cells (Normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV-3, TOV112D, and A2780). Experiments were performed in triplicate. Expression is depicted as the mean with error bars representing standard deviation. All changes in response to talc treatment were significant ($p<0.05$) in all cells as compared to controls.

Figure 5. Epithelial ovarian cancer (EOC) cells have been reported to manifest a persistent pro-oxidant state as evident by the upregulation (green arrows) of key oxidants iNOS, NO, NO+, ONOO-, OH-, O2+, and MPO (blue) and downregulation (red arrows) of key antioxidants SOD,

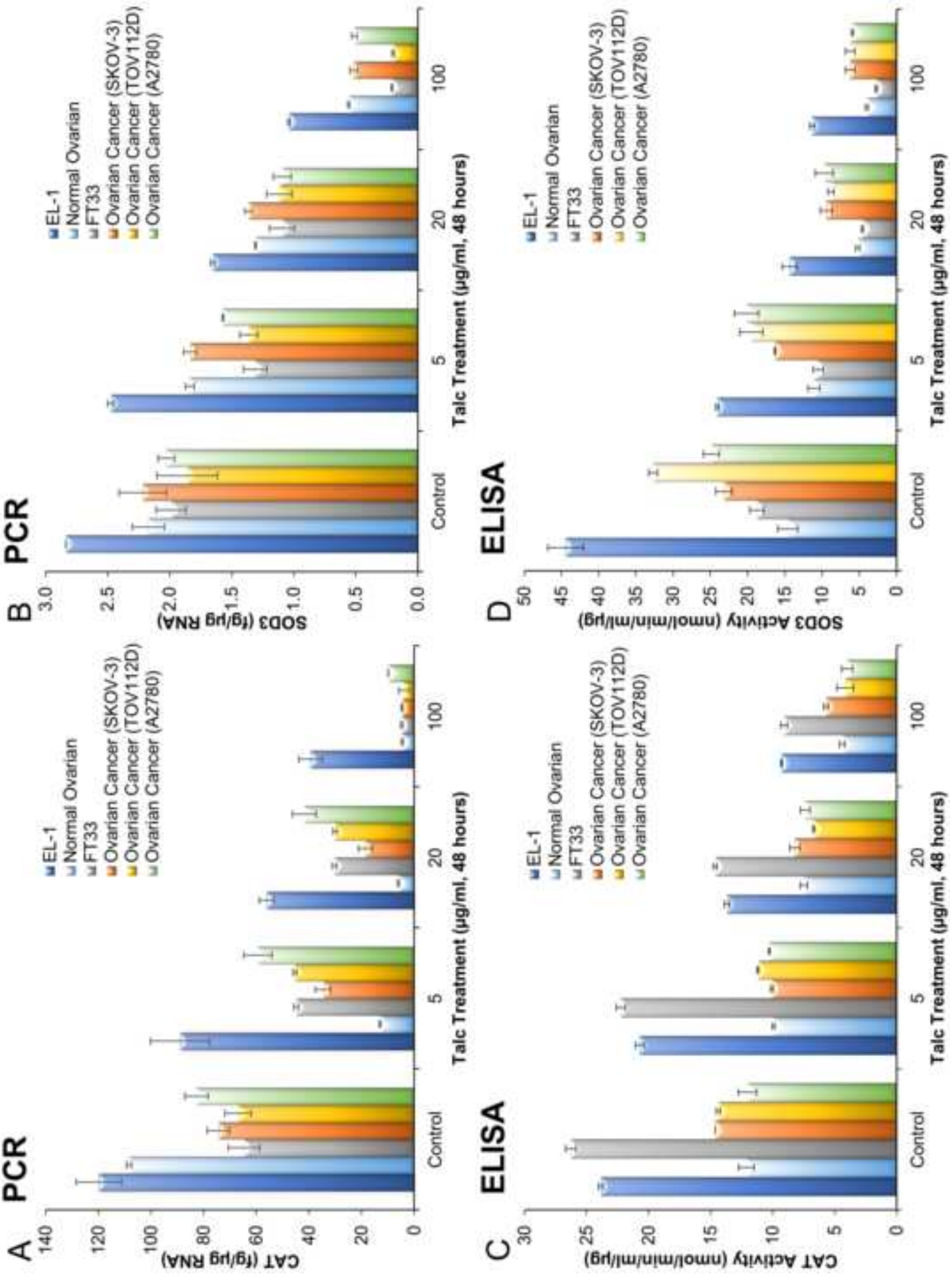
CAT, GPX, and GSR (orange). This redox state was also shown to be further enhanced in chemoresistant EOC cells. In this study, talcum powder altered the redox state, as indicated by the arrows, of both normal and EOC cells to create an enhanced pro-oxidant state.

Supplemental Material Legends

S1. Real-time RT-PCR oligionucleotide primers.

A	Gene (rs number)				
	CAT (rs769217)	*NOS2* (rs2297518)	*GSR* (rs8190955)	*GPX1* (rs3448)	*SOD3* (rs2536512)
MAF	0.123	0.173	0.191	0.176	0.476
SNP	C-262T	C2087T	G201T	C-1040T	A377T
Chromosome Location	11p13	17q11.2	8p12	3q21.31	4p15.2
Amino Acid Switch	Isoleucine to Threonine	Serine to Leucine	Unknown	Unknown	Alanine to threonine
Effect on Activity	Decrease	Increase	Unknown	Unknown	Decrease

B	Gene (rs number)				
Cell Lines	*CAT* (rs769217)	*NOS2* (rs2297518)	*GSR* (rs8190955)	*GPX1* (rs3448)	*SOD3* (rs2536512)
A2780- Control	C/C	C/C	G/G	**C/T**	A/A
A2780- Talc	C/C	C/C	G/G	C/C	A/A
SKOV-3- Control	C/C	C/C	G/G	**C/T**	A/A
SKOV-3- Talc	C/C	**T/T**	G/G	C/C	A/A
TOV112D- Control	C/C	C/C	G/G	**C/T**	A/A
TOV112D-Talc	**C/T**	C/C	G/G	C/C	A/A
HOSEpiC- Control	C/C	C/C	G/G	**C/T**	A/A
HOSEpiC- Talc	**C/T**	**T/T**	G/G	**C/T**	A/A
FT33- Control	C/C	C/C	G/G	**C/T**	A/A
FT33- Talc	**C/T**	**T/T**	G/G	C/C	A/A
Normal Ovarian- Control	C/C	C/C	G/G	**C/T**	A/A
Normal Ovarian- Talc	**C/T**	**T/T**	G/G	C/C	A/A



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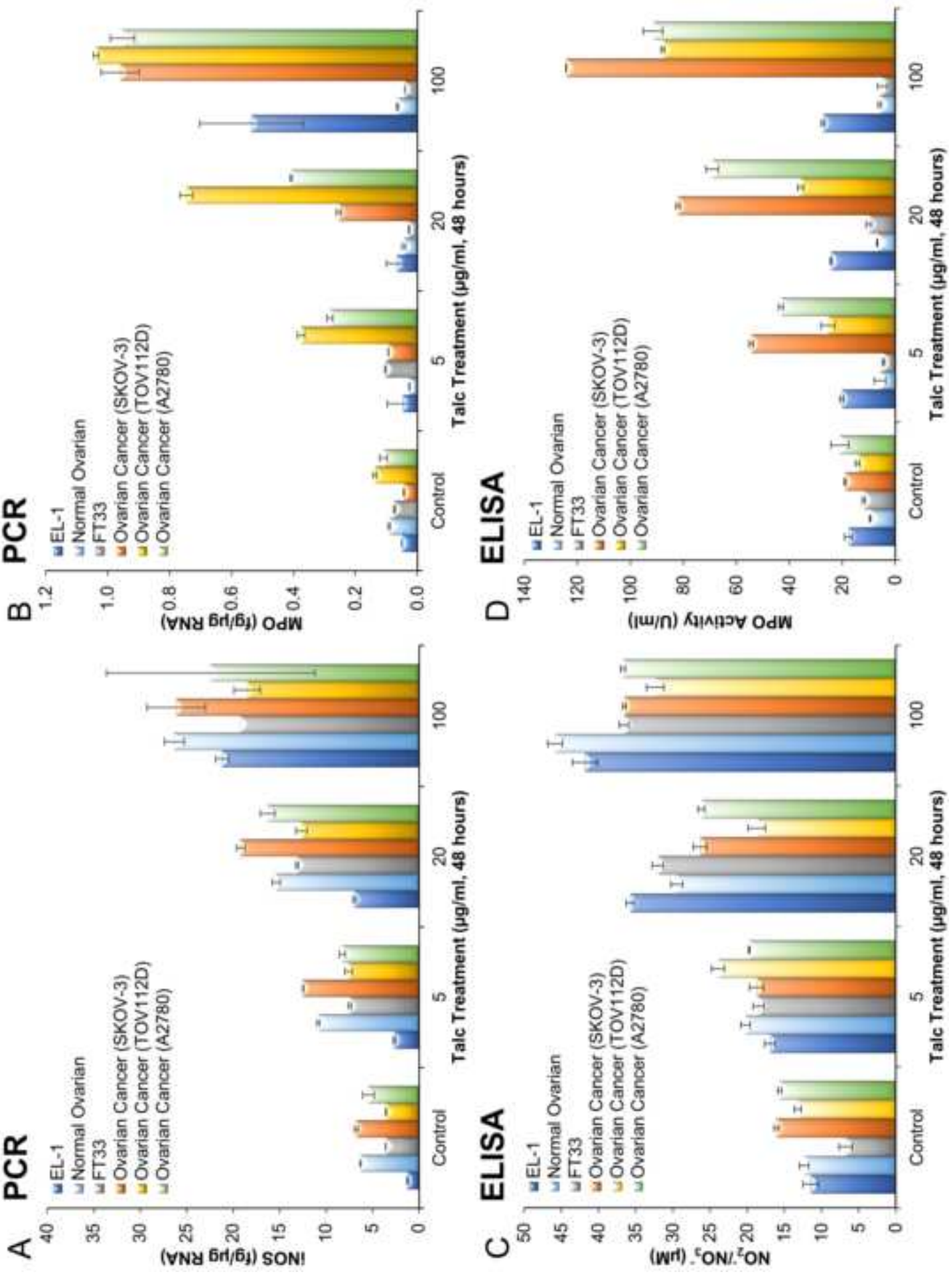

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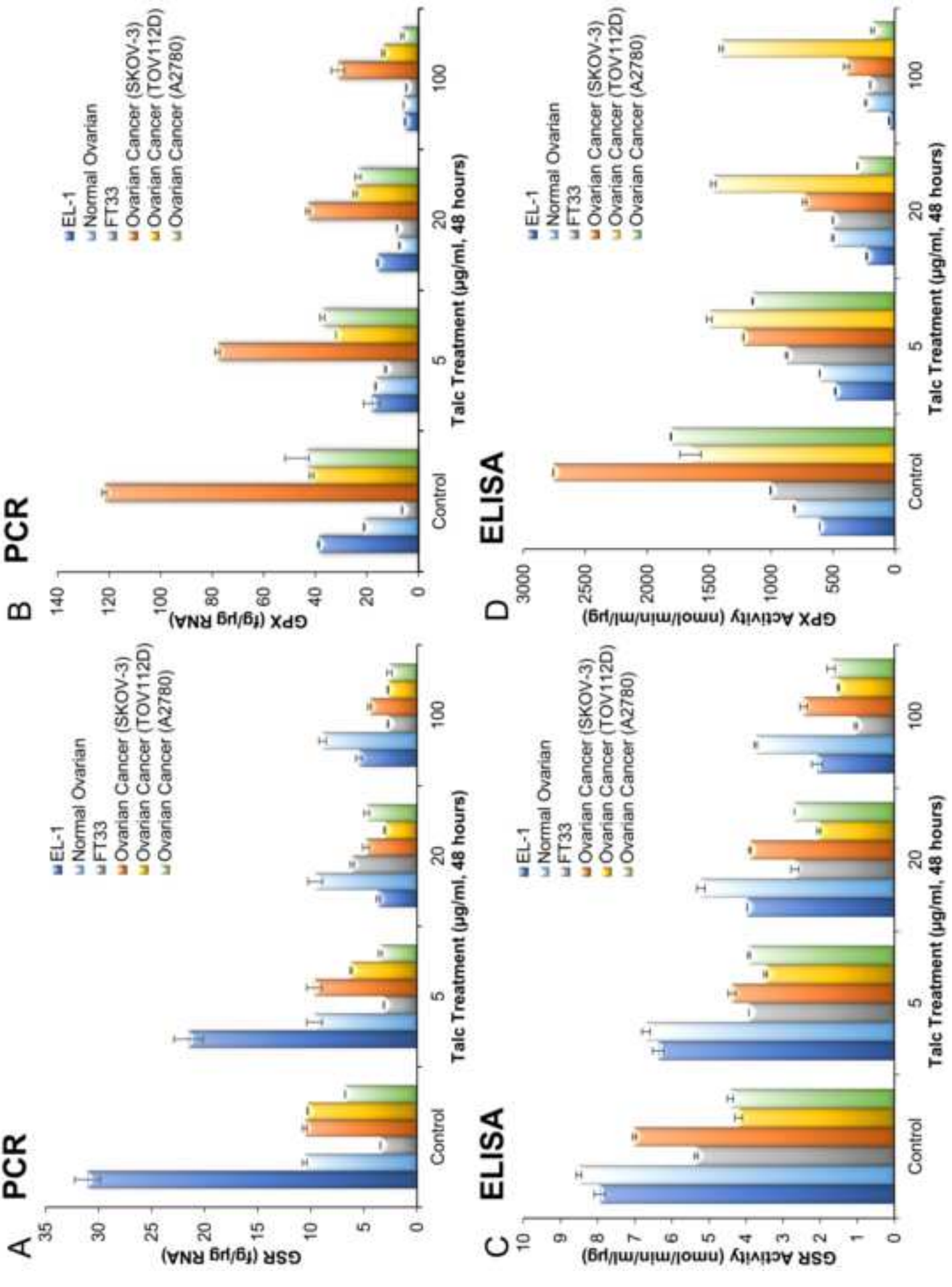

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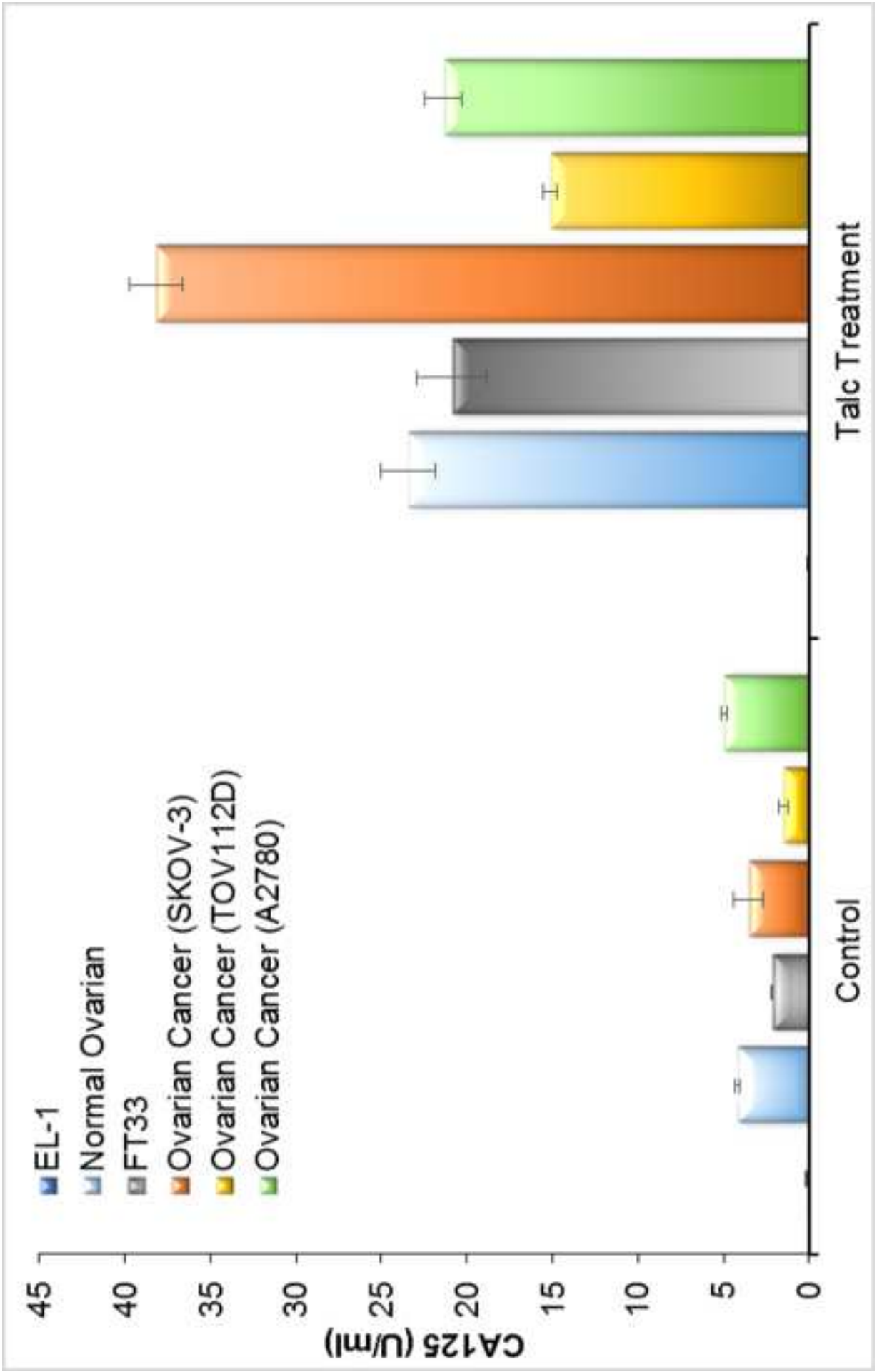

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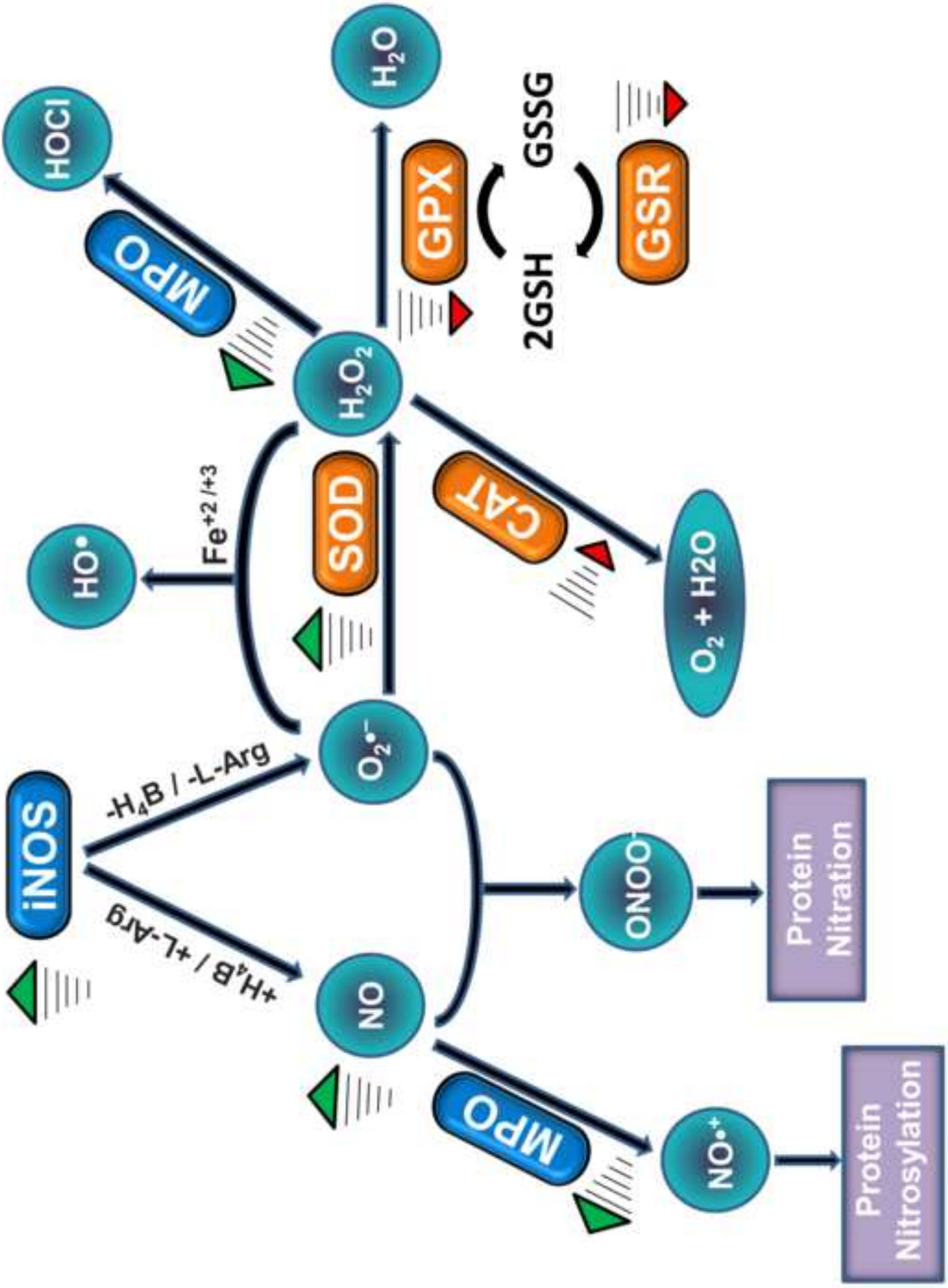

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Accession Number	Gene	Sense (5'-3')	Antisense (3'-5')	Amplicon (bp)	Annealing Time (sec) and Temperature (°C)
NM_001101	*β actin*	ATGACTTAGTTGCGTTACAC	AATAAAGCCATGCCAATCTC	79	10, 64
NM_001752	*CAT*	GGTTGAACAGATAGCCTTC	CGGTGAGTGTCAGGATAG	105	10, 63
NM_003102	*SOD3*	GTGTTCCTGCCTGCTCCT	TCCGCCGAGTCAGAGTTG	84	60, 64
NM_000637	*GSR*	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116	10, 63
NM_000581	*GPX1*	GGACTACACCCAGATGAAC	GAGCCCTTGCGAGGTGTAG	91	10, 66
NM_000625	*NOS2*	GAGGACCACATCTACCAAGGAGGAG	CCAGGCAGGCGGAATAGG	89	30, 59
NM_000250	*MPO*	CACTTGTATCCTCTGGTTCTTCAT	TCTATATGCTTCTCACGCCTAGTA	79	60, 63

1

1 Highlights

- 2 Oxidative stress is a key mechanism to the initiation and progression of ovarian
3 cancer.
- 4 Talcum powder induces key inflammatory and redox markers.
- 5 Talcum powder induces mutations in key oxidant and antioxidant enzymes, which
6 confers its effect.
- 7 Here we demonstrate a mechanism of the cellular effect of talcum powder.